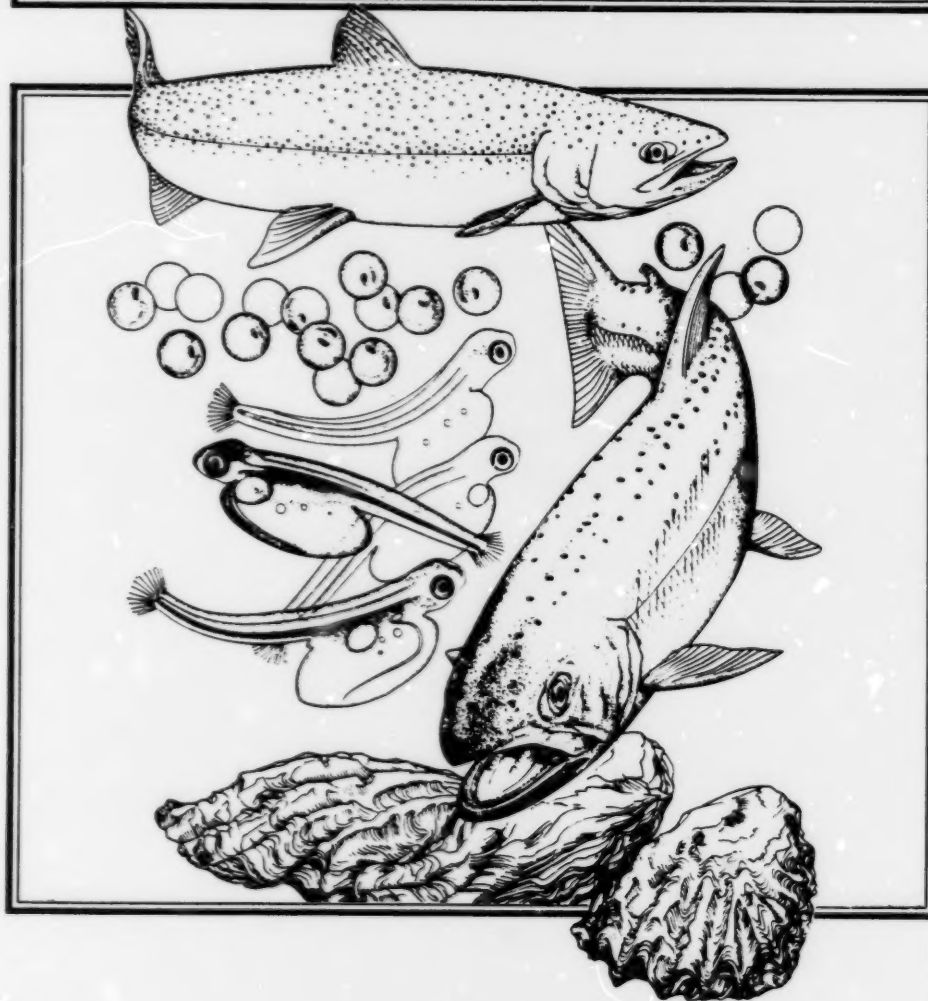


Aquaculture Industry Development Report
Gene Banking for Pacific Salmon:
Technology Assessment and
Applications in British Columbia



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Summary

Collection and banking of sperm of Pacific salmon species will facilitate important management objectives in salmon culture in British Columbia including *programs of genetic improvement of broodstock, conservation of untapped and endangered genetic variability, hybridization between on year/off year runs, fertilization when sperm and eggs are available at different times, and transport of sperm free of time constraints.*

Establishing a bank of salmonid sperm is now possible using *cryopreservation*, a technique in which biological material is maintained indefinitely in a state of suspended animation at the temperature of liquid nitrogen (-196°C). Goals of sperm banking in aquaculture are analogous to those in livestock husbandry, and cryopreservation technology will have an equivalent impact on cultivation and management of aquatic organisms. Whether freezing is done on site or at the bank, several decades of experience with artificial insemination in cattle indicate that centralized storage will be more cost effective and efficient than training and equipping individual farms in the technology.

Present cryopreservation technology for salmonid sperm yields excellent fertility and development in laboratory and field trials but must be scaled up for widespread application in British Columbia. Specific technical problems to be overcome in addition to the general engineering problem of scale-up include *optimizing post-thaw motility, fertility and transport procedures.* Based on presently available technology, solutions to these problems can readily be found through specific short-term research and process engineering using facilities and expertise presently available in the province.

Cryopreservation of salmonid *eggs and embryos* is a highly desirable goal. However, further research in a laboratory with specific cryobiological expertise and equipment is needed before there can be any practical application of this technology in British Columbia.

1. The Need for Gene Banking in Aquaculture

Perpetuating healthy populations of economically important organisms implies *genetic diversity*. This concept is familiar to most people for agricultural crops, where seed banks have been set up to conserve genetic material from important land races threatened by environmental disturbances. The concept is no less important for aquaculture in general, and for Pacific salmon species in particular, now under domestication selection pressure.

The need for conservation of genetic material in aquaculture and fisheries enhancement is now well recognized. Several private companies are interested in establishing banks of fish gametes along the lines of the many highly successful sperm banks for livestock.

Public sector support has taken the form of workshops on genetic conservation and policy statements with particular reference to salmonids. In a national effort in Norway, salmonid sperm is presently being banked for genetic conservation.

In British Columbia, unique races of Pacific salmon represent the genetic "capital" on which both stock enhancement and a successful farming industry must be based. This capital depreciates when salmon runs are endangered by environmental disturbances, and the variability for important production-related traits is still mostly untapped. Many of these populations can be exploited in enhancement efforts and will be of great importance in future selective breeding, and they represent a vast genetic repertoire of adaptation. One fundamental function of a salmon sperm bank will be to permit collection of genetic material from these unique populations to ensure that potentially useful variability is not lost forever.

As selective breeding for enhancement and aquaculture proceeds, this reservoir of variability can be tapped to ensure that loss of fitness in partially domesticated strains is not debilitating and that progressively genetically impoverished populations are not at the mercy of unexpected environmental change. *In the case of many important crop species this variability has already been lost; for aquaculture, an industry just embarking on a period of genetic improvement, the opportunity is still there.*

As attention to a strain's genetic worth becomes an increasingly higher priority in salmon culture and enhancement, a bank of genetic material will enable programs to be designed rationally, based on an inventory of known stocks.

Apart from its role in genetic conservation, cryopreservation of gametes will be a routine and invaluable *practical tool* for hatcheries, allowing them to bank surplus milt, to transport milt reliably between sites, and to perform fertilizations without complete dependence on the timing of natural gamete maturation. (Even greater freedom from gamete maturation timing will be permitted when technology for freezing *embryos* is available, as will much more efficient use of hatchery rearing space).

2. Cryobiology and Cryopreservation

Cryobiology is a branch of biotechnology concerned with the effects of low temperatures on biological systems. Since the 1940s, when spermatozoa of humans and animals were first frozen, advances in cryobiology have profoundly affected human and veterinary reproduction by making possible artificial insemination and In Vitro Fertilization (IVF) programs, and have facilitated preservation and propagation of elite genetic material in agriculture. *Banking and sale of human and animal spermatozoa and embryos is now a multimillion dollar international business, and the aquaculture industry, with its huge demand for high quality and genetically enhanced gametes and embryos free of the time restrictions of natural spawning schedules, is poised to take advantage of the same technology.* Recent developments in cryobiology, particularly in ultrarapid freezing of embryos, point to an explosion in new applications in aquaculture, agriculture and medicine.

In aquaculture as in livestock husbandry, gene banks rely on the technique of *cryopreservation* - freezing and storage at the temperature of liquid nitrogen, -196°C . There appears to be little risk that the aquaculture community, and particularly salmon farmers in B.C., will be any more likely than cattlemen to reject the use of cryopreserved material. Acceptance of the concept of cryopreservation technology is clearly evidenced by the volume of published reports of laboratory scale methods for fish spermatozoa as well as the growing interest of local fish and shellfish farmers in trying out the technology.

3. Specific Applications in British Columbia

Cryopreservation technology for salmonid eggs and embryos lags far behind that for sperm, and presently has no practical importance.

Cryopreservation technology for salmonid sperm facilitates some specific management goals; these are well described by Cloud et al (1990), and can be characterized as *genetic conservation*, *genetic improvement*, and *hatchery management*. As cryopreservation expands, inventories of stocks that have been genetically improved or are believed to offer advantages in culture will build up. These inventories, although small at first, can immediately be used to facilitate the following goals:

3.1 Broodstock improvement and genetic conservation

Permits collecting and introducing genes from wild or native populations of Pacific salmon into hatchery stocks as part of an overall selective breeding program, particularly when times of spawning do not overlap;

Maintains a reservoir of genetic variability so cultured populations are not vulnerable to environmental change such as a new disease;

Permits inventorying characterized milt - either genetically enhanced, such as the sex-reversed chinook salmon milt, or milt from males with a known disease history.

3.2 Hatchery operation

Permits hybridizing "on year" / "off year" runs;

Permits fertilization when sperm and eggs are available at different times or where there may be a shortage of sperm;

Permits reliable transport of sperm free of time constraints that presently limit movement of fresh semen.

4. Cryopreservation Technology: Principles and State of the Art

The scientific literature between 1986 and 1990 contains 25 references to cryopreservation of fish sperm; those that are relevant to Pacific salmon are cited in the bibliography. There is only one reference to experiments on freezing fish embryos. Only one patent has been issued relating to fish spermatozoa, in the Soviet Union for fertilizing ability of frozen carp sperm. This patent is not relevant to cryopreservation of salmonid sperm.

4.1 Steps in cryopreservation

To freeze any biological system there are four specific problems to overcome: to *protect* cells and tissues with a suitable cryoprotectant that minimizes the chances of intracellular ice formation; to *cool* the material to the temperature of liquid nitrogen at a rate that allows desiccation and prevents intracellular ice formation; to *thaw* frozen material to ambient temperature without incurring recrystallization and surface solute effects; and to *remove the cryoprotectant* and demonstrate normal development thereafter.

4.2 Cryoprotectants

When fresh, untreated fish sperm (milt) is cooled below its freezing point, two forms of fatal damage occur: ice forms inside the cell, and the dehydration that takes place during cooling results in membrane damage as the salt concentration outside the cell increases. Both effects can be reduced or eliminated by adding *cryoprotectants* to the milt.

Cryoprotectants are *intracellular* (penetrating) or *extracellular* (non-penetrating). Intracellular cryoprotectants are more important, and include (in rough order of frequency of use) *dimethyl sulphoxide (DMSO)*, *glycerol*, *propylene glycol (propanediol)* and *methanol*. Intracellular cryoprotectants work best at final concentrations between 5 and 10% volume/volume. DMSO at a final concentration of 7.5% is a good all-round cryoprotectant for many cell types; glycerol has worked well in some fishes; methanol works well in some fishes and not in others.

Extracellular cryoprotectants often elevate fertility beyond what can be achieved using an intracellular cryoprotectant alone. They act by coating membranes and preventing salt damage.

4.3 Diluents

Cryoprotectants are added to sperm by way of a vehicle called a *diluent* (sometimes called an *extender* because it has the added advantage of reducing sperm cell density to a number convenient for artificial fertilization). Much effort has gone into developing complex diluents that mimic the composition of seminal plasma, but really the only requirement of a diluent is that it carry the cryoprotectants to the sperm cells and that sperm cells are not activated (become motile) in it. Excellent results are now being obtained by adding cryoprotectants to the simple vehicle of glucose in distilled water. Preventing sperm activation in the diluent is essential because salmonid sperm cells swim for a short period only, and they must do so when they are in contact with eggs, not while they are being readied for freezing. Usually the cryoprotectants themselves inhibit sperm movement.

When milt and diluent are mixed, intracellular cryoprotectants rapidly penetrate the sperm cells, and extracellular cryoprotectants coat the outside of the cell. The ratio of diluent to milt determines the number of sperm cells in a given volume, and can be adjusted to a density that works well for artificial fertilization. When thawed sperm is added to eggs with a little water, intracellular cryoprotectants are diluted out to levels that are not toxic to eggs or sperm.

4.4 Biology of freezing and thawing

When protected sperm cells are *cooled* the surrounding medium freezes at a few degrees below 0°C. As cooling continues, water leaves the sperm cells to balance the free water "lost" when the medium froze. If cooling is not too fast, the cells dehydrate as the temperature falls, and ice never forms inside the cell. By about -50°C the cells are shrunken, the danger of ice forming inside the cells is past, and cooling can be faster.

When frozen sperm is *warmed*, ice formed outside the cells thaws and water re-enters the cells. *In most cases a proportion of the sperm cells begin to swim spontaneously, even though they were not motile before freezing.* The fertilizing ability of the thawed sperm is not affected but, because the duration of motility is short, thawed milt must be added to eggs without delay.

4.5 Cooling and warming rates

Fish spermatozoa should be cooled at between about 10 and 50°C/minute, and thawed as rapidly as possible. Cooling and thawing rates depend on the container used for freezing, and one container that gives consistent results is the *plastic straw* used to freeze cattle semen (0.25 or 0.5 ml). Pellet or droplet freezing in depressions on dry ice also works but is hard to standardize and of course requires dry ice. Straws, on the other hand, can be laid on a hand-made wire rack held over liquid nitrogen in an insulating container; as long as the container is allowed to equilibrate, the distance above the liquid surface determines the cooling rate, and rates are repeatable.

4.6 Shelf life and storage containers

Biological material stored in liquid nitrogen remains viable indefinitely, and can be thawed out and used many years after it was frozen. Spermatozoa are no exception, and should be thawed only when fresh eggs are waiting to be fertilized. The amount of frozen sperm to use is based on whatever is commonly done with fresh milt. The process of fertilization is the same as with fresh milt. In studies where fertilization, development and larval performance have been measured, cryopreserved sperm has proven as effective as fresh sperm, with no increase in abnormalities.

Frozen fish sperm is best stored in a standard liquid nitrogen refrigerator, commonly used for storage of animal and plant cells, sperm and embryos. Refrigerators are available from several manufacturers in various sizes and capacities; maintenance is limited to periodic topping up with liquid nitrogen, and alarm systems are available for detecting low nitrogen levels.

4.7 Quality control

Batches of frozen salmonid sperm need to be characterized as to predicted fertility. However, the long production cycle in salmonids makes it impractical to monitor test fertilizations even to the eyed stage. A great deal of contradictory material has been written about the relationship between sperm *motility* and *fertility*, and there are certainly cases where eggs appear to have been fertilized by non-motile sperm. In practical terms, however, vigorous motility goes along with fertility. Salmonid sperm motility, although short-lived, can be estimated using standardized techniques in an aliquot of each batch of frozen sperm, and used with confidence as a predictor of viability.

4.8 Importance of gamete quality

The eggs of some females, and the milt of some males, are more fertile than others. Sperm frozen early in the spawning season is usually more fertile than sperm frozen at the end of spawning. Egg quality can also strongly affect the outcome of a fertilization, and must be standardized by using eggs from the same female whenever sperm treatments are compared. Sperm quality also deteriorates after removal from the fish, but there is no clear trend that might recommend immediate freezing over chilling the milt for variable lengths of time before freezing. The safest course is to standardize the delay between extraction and freezing.

5. Evaluation of Present Technology for Salmonid Milt

Researchers with a practical interest in storing fish sperm have been developing laboratory-scale cryopreservation methods for the past decade, with the emphasis on salmonids and important tropical farmed species. Notable advances during this period have come from Stoss (for example, Stoss and Holtz 1983; Stoss and Refstie 1984) and Harvey (for example, Harvey 1982; 1983; 1987; Harvey and Kelley 1988). Munkittrick and Moccia (1984) review research results and potential applications to salmonid culture. Fish sperm has been frozen successfully using adaptations of the "pellet technique" and the "straw technique" from livestock husbandry.

Fertility using salmon sperm frozen by either technique is generally good, although variations have occurred because procedures for fertilization with thawed sperm have been patterned on those using fresh milt and do not take into account the alteration of membrane permeability and consequent heightened osmotic sensitivity that accompanies cryopreservation. These variations could be eliminated so long as a sufficient supply of eggs is available for testing.

All methods presently used are too small scale to be useful in preserving the large volumes of milt required in B.C. They are geared to multiple units of 0.25-0.5 ml, an amount far too small to be used in practice where thousands of eggs must be fertilized at the same time.

5.1 The pellet method

Pellet freezing of sperm is done by forming droplets in depressions made in a block of dry ice. The droplets, once frozen into pellets, are transferred to liquid nitrogen for storage. Pellets are thawed by dropping them in a thawing solution, which is then mixed with eggs. The pellet method for salmon sperm was developed by Stoss and colleagues at Fisheries and Oceans Canada's West Vancouver Laboratory in the early 1980s.

The pellet method, while useful on a laboratory scale, is unsuited to scale-up for the volumes of sperm that must be stored in B.C. Because the sperm is not frozen in a container such as a vial or tube or straw, the only way to freeze larger volumes is to freeze more droplets. Changing droplet size will unfortunately change the cooling and thawing rates, which are critical for survival. Freezing more droplets means making more depressions in more blocks of dry ice and filling each manually. A simple calculation shows that to freeze the milt from one male (say 50 ml), semen extended 1:1 with cryoprotectant would require 200 droplets.

Other drawbacks to the pellet method are the need for two coolants (both dry ice and nitrogen for storage are needed; material stored on dry ice has a somewhat shorter shelf life). The practical handling and organization of large numbers of pellets is also a problem: pellets must be grouped according to donor, which implies sealing them within a container that is then stored in liquid nitrogen. Present nitrogen storage containers are designed to hold material frozen in straws and, again because of the large volumes of salmon sperm that need to be stored, efficient use must be made of all containers.

Pellet freezing had some application several decades ago for livestock, but has since been superseded by the use of straws. This level of technology is untenable where litres of fish sperm must eventually be frozen.

5.2 The straw method

Semen straws are 0.25 or 0.5 ml plastic tubes made specifically for freezing semen and so manufactured as to be sealable at both ends. Filling, sealing and labelling can be automated, and cooling machines are available to handle large numbers of straws. Straw freezing is now almost universal for conservation of livestock gametes (ova are also frozen in straws), and has replaced pellet freezing wherever volume, efficient storage and elimination of cross-contamination are necessary.

The method is excellent for mammalian sperm, where one 0.5 ml straw is sufficient to fertilize one female. Salmon sperm can also be frozen in straws, with the only drawback being the requirement for large numbers of straws to handle the large volumes of milt. Chinook salmon milt, both normal and sex reversed, can be frozen in straws after dilution in a simple extender using the cryoprotectant dimethylsulfoxide (DMSO). Post thaw fertility varies between 20% and 80%, and more needs to be known about the best technique for performing fertilizations with frozen-thawed sperm.

5.3 Future developments

The use of cryopreserved sperm in aquaculture will expand dramatically as we learn more about the control of sperm motility. At present we know motility is inhibited by high potassium concentrations in the testis and is initiated by potassium dilution. Studies on sperm physiology are now beginning to show us how to prolong and even to reactivate motility after sperm have stopped swimming, and the results of these studies will further simplify cryopreservation techniques.

6. Criteria For a Practical Cryopreservation Technique and Problems Needing Solution

6.1 General requirements

A cryopreservation technique useful for collection of sperm for broodstock management and development in British Columbia must fulfill several criteria:

- A It must be possible to freeze and thaw sperm without using costly, specialized equipment (such as controlled rate cooling machines) that cannot easily be transported to a remote site;
- B It must be possible to freeze and to thaw realistic amounts of semen (in the case of Pacific salmon, 5 ml samples);

- C Freezing containers must be easily marked and stored without specialized equipment (such as automated labellers widely used for livestock semen);
- D It must be possible to transport the frozen semen inexpensively to and from the hatchery or stream;
- E Sperm viability, expressed as motility after thawing and activation, must be at least 50%;
- F Fertility of frozen-thawed spermatozoa must be greater than 80%, and development to eyed stage at least 70%;
- G The freezing method must provide acceptable post thaw motility and fertility with sperm up to 24 hours old, to allow for transportation time from remote sites.

In summary, it must be easy and inexpensive to freeze, label, store, keep track of, ship, retrieve, thaw and fertilize with salmon sperm. Based on presently available technology, all of these requirements can be filled through specific short-term research combined with process engineering. Solution of the specific problems described below can readily be achieved using facilities and expertise presently available in the province.

6.2 Specific problems to solve before implementing the technology

6.2.1 Scale-up and process engineering

What is now needed is to develop freezing containers with 5 ml capacity that can be labelled, frozen without specialized rate-controlling equipment, stored efficiently in existing nitrogen refrigerators, and easily retrieved and thawed. Scale-up is a relatively straightforward matter of manipulating container size and shape to achieve the required rates.

6.2.2 Centralized vs on-site freezing and storage

Development of 5 ml freezing containers allows the flexibility of freezing on site or at a central freezing and storage depot. A useful technique for freezing, storing and fertilizing with salmon sperm has to take into account the logistics of collection as well as acceptability by hatchery workers. Thus, while it is possible right now for hatchery technicians to freeze milt, most will not have the time for training in the new procedure. A major objective before implementing the technology on a large scale is to decide whether logistics and training allow the process to be done *on site*, or whether it is better to transport fresh, chilled milt to a *central depot* for processing.

Centralized freezing will succeed if two criteria are met: transport costs must be affordable, and transport time for fresh milt must not be too great. Present experience with freezing "aging" salmon sperm suggests that 8-12 hours transit time on ice is acceptable. This assumption has to be tested before the technology is applied. *Once it is proven that fresh sperm can be frozen after a delay for transport, the most efficient way to implement the technology in British Columbia is to contract the services of a centralized sperm bank that will receive, freeze, catalogue, store, thaw and ship milt from public and private clients.* If transport delay proves unacceptable, freezing can be done on site as is presently done in Norway.

Whether freezing is done on site or at the bank, several decades of experience with artificial insemination in cattle indicate that centralized storage will be more cost effective and efficient than training and equipping individual farms in the technology.

6.2.3 Optimizing motility

A major objective in freezing salmon spermatozoa is to extend the *pre-activated* viability of fresh sperm and of frozen-thawed cells so that fertilization need not be tightly tied to thawing; extended viability will allow shipping of fresh milt to a central freezing facility as well as shipping of thawed milt to a remote site for fertilization. Post-thaw motility should also be extended to 60 seconds to permit ease in fertilization. The approach taken should build on recent advances in the control of salmon spermatozoan motility (see, for example, Christen et al., 1987, Eur. J. Biochem 166: 667-671).

7. Freezing Salmon Eggs and Embryos

Salmon embryos present an unusual technical challenge by virtue of their size and architecture. While relatively advanced embryos - eg. sea urchin and oyster - can be frozen successfully and degree of tissue complexity is not the automatic bar to success it was once considered, the problem with salmon lies in the volume of yolk and the relative osmotic isolation provided by the chorion (egg membrane). Attempts to cryopreserve the embryos of salmon and other fishes are described by Harvey and Ashwood-Smith (1982), Harvey (1983), Harvey et al (1983; 1984). The technology could be developed over a period of several years, with the research taking place at a well equipped cryobiology laboratory. To be useful in culture of Pacific salmon, embryos could be frozen at any stage post fertilization but survival of thawed embryos to hatching must be at least 50%, and cryoprotectant chemicals, if successful in preserving embryos, must be demonstrated not to cause genetic effects.

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